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14. ABSTRACT
This study will recruit wounded warriors with severe extremity trauma, which places them at high risk for heterotopic ossification (HO); bone formation at abnormal sites, which causes pain, limits motion and/or limits the use of a prosthetic device. There are three goals: 1) to understand the mechanisms involved in HO; 2) to define accurate and practical methods to predict where HO will develop; and 3) to define potential therapies for prevention or mitigation of HO.

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INTRODUCTION

The mechanism(s) involved in heterotopic ossification (HO) in our severely injured wounded warriors are unclear. Accurate, practical methods and assessment tools (macroscopic and cellular/molecular) need to be developed to characterize wounded tissues and predict where HO may or will develop. These tools need to provide insight into the biological wound environment and events that contribute to or elicit HO. These tools also need to provide effective methods for early diagnosis or risk assessment (prediction) so that therapies for prevention or mitigation of HO can be optimally targeted. This study seeks to contribute to advancement in each of these key areas.

The research teams at Cleveland Clinic Lerner Research Institute (CC), Walter Reed National Military Medical Center (WRNMMC) and Naval Medical Research Center(NMRC), bring together robust and complimentary experiences. The research team at CC performs quantitative wound assessment using non-invasive imaging modalities (ultrasound), *in vitro* assay and characterization of tissue-resident connective tissue progenitors (CTPs) using image analysis of colony forming unit performance, and the teams at WRNMMC and NMRC perform Raman Spectroscopy and gene expression profiling in at-risk tissue from HO+ and HO- patients.

Year 2 work focused on sample processing, data collection and finalizing data tables required to capture study data from the time of patient enrollment through to final sample analyses. Collecting tissue samples from patient 01 gave team members an opportunity to review the flow process for tissue samples, patient information, and data sharing. Dr. Davis's lab (NMRC) shipped slides of patient 01's cultured wound site samples to the Muschler laboratory (CC). The Muschler lab performed colony analysis on these samples, providing cell and colony data that was shared with team members. H&E stained biopsied samples (NMRC) were scanned (CC) and viewed for qualitative assessment and data tables with gene transcript analysis (NMRC) of biopsied wound site samples were viewed and discussed during bi-monthly team meetings. The Luminex assay was done on serum and effluent samples from patients 01 and 02 (WRNMMC). Patient 02's slides of cultured wound site samples have now been shipped from (NMRC) and received by the (CC) for cell and colony analysis. Gene transcript analysis has been completed from eight total biopsied sites (4 per wound), of which all (8/8) subsequently developed radiographic evidence of HO. These two patient enrollments have provided three total extremity wounds for analysis.

Between 10 June 2013 and the present, the incidence of transfemoral injury has fortunately been significantly less than that seen in recent years. Initial inclusion criteria has been modified to now include both below knee and upper extremity amputations and also includes severe military limb trauma with >75 cm^2 area of open tissue. In this population, these wounds are also at risk for HO, though less than that seen in the setting of transfemoral amputations and hip disarticulation. We have formally proposed a no cost extension to enroll at least a total of 5 additional subjects, In addition, we submitted a proposal to extend our assessment tools using a well characterized rat blast overpressure amputation model that incorporates the critical elements associated with combat-injury specifically a blast injury, femur fracture-crush and transfemoral amputation through the zone of injury wherein all animals develop radiographic evidence of HO within 2 months post-injury

Year 2 Refinements include:

.Colony Analysis-(NMRC-CC)

Dr Davis's laboratory (NMRC) processed biopsied wound site samples collected on Patients 01 and 02. The samples were set at 10,000, 15,000 and 20,000 cells /ml at oxygen tensions 0.3%, 3%, and 20%. These slides were harvested by fixation at day 6 and shipped to the Cleveland Clinic for colony analysis. The Muschler Laboratory (CC) received 8 boxes of slides with 18 slides per box for a total of 144 slides for each patient. For patient 01 samples, all oxygen tensions at plating densities 10,000 and 15,000 cells/ml were stained and scanned for Colonyze™ processing. Colony prevalence data for these samples was calculated and shared with team members during a team teleconference. Alkaline phosphatase analysis on these samples is in progress. Patient 02 samples are being stained and processed for colony analysis.

Labeling Protocol

A slide label convention has been developed that defines eight (8) key variables on each slide for tracking HO+ and HO- patient samples shipped from NMRC to CC.

Gene Array (NMRC)

A custom gene array for assessing adipogenic, chondrogenic, osteogenic, angiogenic and wound healing mRNA transcripts has been developed. Biopsied wound sites were analyzed based on either a 4 fold increase or 4 fold decrease, and the data was presented as genes over expressed or under expressed in injured muscle vs control muscle. In patient 01, when examining gene expression among a series of the 84 gene transcripts, it was found that site 3 was distinctly different than the other three sites. This included 10 fold or more lower expression levels in several markers associated with bone differentiation (Adipoq, BMP4, Col4a3, IGF2, MyoD1, Smad3); 3-20 fold lower elevation of expression of Col1a1; and 3-10 fold higher expression of MMP9 and Spp1. Gene transcript expression analysis for both wounds from Patient 02 has been completed, however assessment between HO^{+} and HO^{-} cannot be made as all eight sites subsequently developed HO

Histology Analysis-(NMRC-CC)

Histoserv, Inc. received human samples from NMRC and processed this tissue to provide histology samples for H&E and Masson's trichrome stain, and immunostain for CD3, CD14 and MPO. Unstained sections were also provided for shipment to CC for tissue HA analysis. Unstained Patient 01 slides were received by CC.

Ultrasound -(CC-WRNMMC)

A test of ultrasound acquired files and transfer to CC was confirmed with the assistance of Mr. Fred Gage, Dr. Jonathan Forsberg, Dr. Trevor Brown and Dr. Felipe Lisboa. Dr. Russell Fedewa completed the validation of the ultrasound data collection SOP.

Raman Spectroscopy (WRNMMC)

An SOP for *in vivo* RS of injured muscle and pre-HO tissue is in place.

Data Storage and Sharing-(WRNMMC-NMRC)

Key elements of data sharing across participating institutions have been addressed. Over 300 variables were considered.

- Sharing includes only de-identified clinical data, wound descriptors, cell harvest and plating details, and CTP quantification and characterization.
- A SharePoint site hosted by WRNMMC has been established for the dedicated use of this study under the Regenerative Medicine Department at NMRC and access from the WRNMMC and CCLRI has been secured. SharePoint access is password protected per user and controlled by defined user roles.
- Data will be stored in Microsoft Access databases with separate data entry forms for each study site. Version control has been established via SharePoint utilities requiring databases to be "checked out" prior to editing.
- Data tables required to capture study data at each step - from patient enrollment through final sample analyses have been finalized. The interface between the individual, site-specific Access databases and the common, centralized database will require revision. Remaining tasks will not hinder the progress of the study at this time and will in no way affect patient care or data quality.

KEY RESEARCH ACCOMPLISHMENTS

- All necessary methods and SOPs have been established and validated, as outlined above and the collection and sharing of data for analysis was finalized. The development and validation of these integrated SOPs provides experience and methods that can be applied to future collaborative projects involving WRNMMC, NMRC and CC.

- A SharePoint site hosted by the WRNMMC and NMRC has been established for the dedicated use of this study under the Regenerative Medicine Department at NMRC and access from all sites has been secured.
- An integrated database has been designed and is ready for data entry for each step from patient enrollment to final sample analysis.
- Gene Expression analysis (GEA) has been completed on biopsied wound samples from patients 01 and 02.
- Serum proteomic measurements for inflammatory mediators (using multianalyte Luminex technology) was performed on serum and effluent samples collected from patients 01 and 02.

REPORTABLE OUTCOMES

- In patient 01's four wound sampling sites, radiographic evidence of heterotopic ossification was found to involve site #1 at 1 month from injury, sites #1,#2, and#4 at 2 months from injury and sites #1,#2, and #4 at 3 months from injury. All confirmed by computerized tomography (CT scan).
- Of the four sites that were biopsied (1,2,3,4) for Patient 01, sites 1,2 and 4 went on to develop HO.
- In patient 01, when examining gene expression among a series of the 84 gene transcripts, it was found that site #3, which did not develop HO, had distinctly lower level of key osteogenic (bone development) gene transcripts than the other three sites.

CONCLUSION

The data from patient 01 offers substantial hope that a rational pattern or combination of gene expression might be used to predict HO occurrence. Of the four sites that were biopsied (1,2,3,4) only site 3 did not go on to develop HO. Gene expression among a series of the 84 gene transcripts, indicate site 3 was distinctly different than the other three sites. If future patients show a similar pattern of gene expression changes, differentiating between future HO sites compared to non-HO sites, may provide important clinical implications in as few as 5-7 patients. We continue to actively screen new patients presenting at WRNMMC.

APPENDICES

Adobe Acrobat Document

Adobe Acrobat Document

Figure 1 Flow Chart Table 1 Osteo MSCs

HO-Quad Chart-10-29-14.ppt

		Development/Cell Signaling Pathways							
Symbol	**Gene Name**	**Osteo**	**Angio**	**Adipo**	**Chondro**	**Myo**	**MSC**	**Inflam**	**Housekeeping**
ACAN	aggrecan								
ADIPOQ	adiponectin, C1Q and collagen domain containing								
ADIPOR1	adiponectin receptor 1								
ALPL	alkaline phosphatase, liver/bone/kidney								
ANGPT2	angiopoietin-2								
BMP2	bone morphogenetic protein 2								
BMP4	bone morphogenetic protein 4								
BMP6	bone morphogenetic protein 6								
BSP	bone sialoprotein								
CD44	*CD44* molecule (Indian blood group)								
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha								
COL10A1	collagen, type X, alpha 1								
COL11A1	collagen, type XI, alpha 1								
COL1A1	collagen, type I, alpha 1								
COL2A1	collagen, type II, alpha 1								
COL4A3	collagen, type IV, alpha 3								
COMP	cartilage oligomeric matrix protein								
CSF3	colony stimulating factor 3 (granulocyte)								
CXCL1 (GRO)	chemokine (C-X-C motif) ligand 1								
CXCL10 (IP-10)	chemokine (C-X-C motif) ligand *10*								
CXCL12 (SDF-1)	chemokine (C-X-C motif) ligand 12								
CXCXL5 (ENA-7	chemokine (C-X-C motif) ligand 5								
ENG	endoglin								
FADP4	fatty acid binding protein 4, adipocyte								
FGF1	fibroblast growth factor 1 (acidic)								
FGF10	fibroblast growth factor 10								
FGF2	fibroblast growth factor 2 (basic)								
FLT1	fms-related tyrosine kinase 1 (VEGFR)								
GLi2	GLI family zinc finger 2								
HAS1	hyaluronan synthase 1								
HAS2	hyaluronan synthase 2								
HAT1	histone acetyltransferase 1								
HDAC1	histone deacetylase 1								
HIF1a	hypoxia inducible factor *1 , alpha* subunit								
HNF1A	HNF1 homeobox A								
IGF2	insulin-like growth factor 2								
IL-10	interleukin 10								
IL-1B	interleukin 1, beta								
IL-6	interleukin 6 (interferon, beta 2)								
IL-8/CXCL8	interleukin 8								
ITGA1	integrin, alpha 1								
ITGA2	integrin, alpha 2								
ITGAM	integrin, alpha M								
ITGAV	integrin, alpha V (vitronectin receptor)								
ITGAX	integrin, alpha X								
JAG1	jagged 1								
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)								
LEP	lLeptin								
LRP5	low density lipoprotein receptor-related protein 5								
MCP-1 (CCL2)	monocyte chemoattractant protein 1								
MIP-1a (CXCl3)	chemokine (C-C motif) ligand 3								
MMP9	matrix metallopeptidase 9								
MYOD1	myogenic differentiation 1								
NOTCH1	notch 1								
OCN	osteocalcin								
OCT4	octamer-binding transcription factor 4								
OMD	osteomodulin								
OPN	osteopontin								
PDGFA	platelet-derived growth factor alpha								
PHEX	phosphate regulating endopeptidase homolog, X-linked								
PPARG	peroxisome proliferator-activated receptor gamma								
PTCH1	patched 1								
PTK2	*PTK2* protein tyrosine kinase 2								
RHOA	ras homolog gene family, member A								
RUNX2 (Cbfa1)	runt-related transcription factor 2								
SCARB1	Scavenger receptor class B member 1								
SMO	smoothened, frizzled family receptor								
SMURF1	SMAD specific E3 ubiquitin protein ligase 1								
SMURF2	SMAD specific E3 ubiquitin protein ligase 2								
SOX2	SRY (sex determining region Y)-box 2								
SOX9	SRY (sex determining region Y)-box 9								
SP1	*Sp1* transcription factor								
Sp7 (OSX)	Sp7 transcription factor (Osterix)								
SPARC	secreted protein, acidic, cysteine-rich (*osteonectin*)								
TBX5	T-box 5								
TERT	telomerase reverse transcriptase								
TGFB1	transforming growth factor, beta 1								
TGFB3	transforming growth factor, beta 3								
TNF-a	tumor necrosis factor								
TWIST1	twist homolog 1								
VEGF-A	vascular endothelial growth factor A								
WNT5a	wingless-type MMTV integration site family, member 5A								
GUSB	glucuronidase, beta								
ACTB	Actin, beta								
B2M	beta-2-microglobulin								
GAPDH	glyceraldehyde-3-phosphate dehydrogenase								
HPRT1	hypoxanthine phosphoribosyltransferase 1								
RPL13A	ribosomal protein L13a								

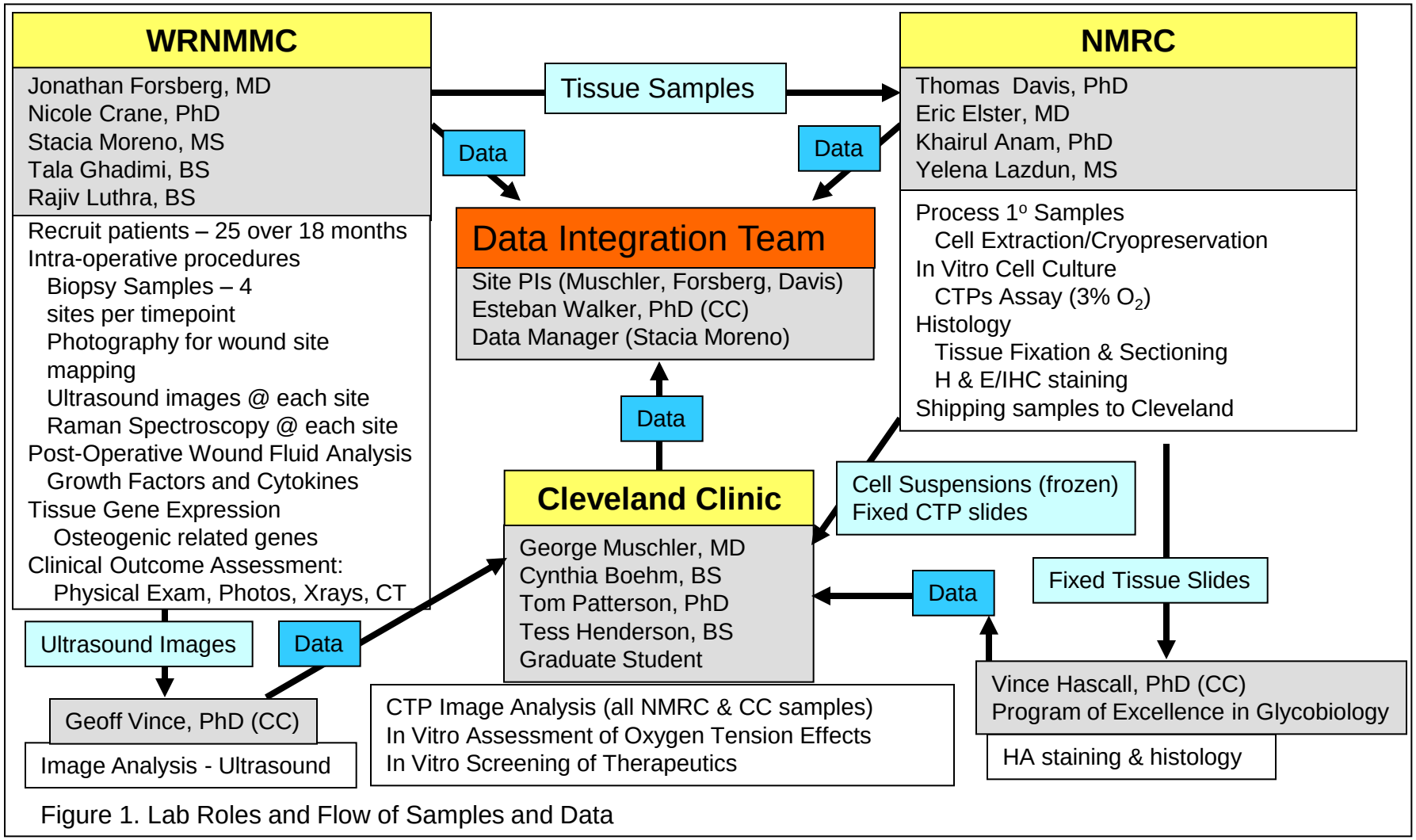

Figure 1. Lab Roles and Flow of Samples and Data